

In Vivo and *In Vitro* Cytotoxicity and Mutagenicity Considerations of Poly (Amido Amine) Dendrimer

Babak Shahbazi^a, Mazaher Khodabandehloo^{a,b}, Mohammad Jafar Rezaei^{b,c}, Samaneh Rouhi^{a,b,d}, Rashid Ramazanzadeh^{a,b*}, Majid Rezaei Basiri^e

^a Department of Microbiology, Kurdistan University of Medical Sciences, Sanandaj, Iran.

^b Cellular & Molecular Research Center, Kurdistan University of Medical Sciences, Sanandaj, Iran.

^c Department of Anatomy, Kurdistan University of Medical Sciences, Sanandaj, Iran.

^d Student Research Committee, Kurdistan University of Medical Sciences, Sanandaj, Iran.

^e Department of Toxicology, Tabriz University of Medical Sciences, Tabriz, Iran.

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ABSTRACT

A lot of chemicals such as poly (amidoamine) (PAMAM-NH₂) dendrimers have pharmaceutical applications, but the major problem with PAMAM-NH₂ is their cytotoxicity and mutagenicity. In this research, we have investigated the cytotoxicity and mutagenicity of various generations of PAMAM-NH₂ (G2.0, G3.0, G4.0, and G5.0). The cytotoxicity of PAMAM-NH₂ at the dilutions of 0.01, 0.001 and 0.0001(W/W) to human mesenchymal stem cells (MSCs) and human gastric adenocarcinoma (AGS) cells was determined using the standard methyl-thiazol-tetrazolium (MTT) assay. To determine mean lethal dose (LD₅₀) of PAMAM-NH₂ at doses of 30, 47, 73.5, 115 and 180 mg/kg, 125 Bagg albino/c (BALB/c) mice (8–10 weeks of age, weighing approximately 20 g) were used and also, for determining the mutagenicity effect of PAMAM-NH₂, 50µL volume of this substance in the Ame's test with *S. typhimurium* was applied. In the MTT assay the most toxic effects, on both of the cell lines, were related to the time when G2.0, G3.0, G4.0 and G5.0 were applied at different dilution of 0.01, 0.001, 0.0001(W/W), respectively. LD₅₀ was determined 73.5 mg/kg. Also in the Ame's test, the number of reverted colonies was increased by applying higher generations and inhibition percentages of PAMAM-NH₂ that were 69.47%, 68.42%, 64.210% and 64.21% for G2.0, G3.0, G4.0 and G5.0, respectively. According to these results, PAMAM-NH₂ generations had cytotoxicity effects on MSCs and AGS cells; also toxicity and mutagenicity of the substance were proved in mice and *S. typhimurium*, respectively. So in order to use PAMAM-NH₂ in pharmaceuticals, it must be subjected to various tests to ensure its safety.

*Corresponding Author: Rashid Ramazanzadeh, E-mail: atrop_t51@yahoo.com, rashid@muk.ac.ir

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Introduction

Because of the low toxicity of nanoparticles, they are used in medical diagnoses, treatment of diseases and antimicrobial therapy [1 - 4]. For example, poly amidoamine (PAMAM-NH₂) dendrimers are nanoparticles that are used in medicine [2]. Dendrimers are a class of hyperbranched, nanoscale macromolecules. All of these materials are originated from a single nucleus and they are described as three-dimensional macromolecules with branching structures. They have a regular structure, high branch, multiple equivalent terminal groups and space between their branches. Empty spaces in dendrimers' structure can accept guest molecules, so encapsulation of particles in different sizes is possible for them. For this reason, scientists have used this material in the treatment of tumors, cancer cells and viral and bacterial infections [5 -7]. Identification of chemical mutagens has become an important procedure in ensuring drug safety [8, 9]. The *Salmonella typhimurium* (*S. typhimurium*) microsome assay (Ame's test) is a widely accepted short-term bacterial assay or a biological test, for detection of mutagenic potential of chemical compounds and identifying substances that can cause genetic damage [10, 11]. Also the methyl-thiazol-tetrazolium (MTT) assay is a method for cell viability recognition and is applied in biological labs [12]. Determining the mean lethal dose (LD₅₀) of a material is finding a dose that is required for killing 50% of the tested animals after testing certain chemicals on them [13]. Different studies have shown cell viability,

cytotoxicity and mutagenicity of different materials using MTT assay, LD₅₀ and Ame's test: Nam et al. in Korea by utilizing the MTT assay showed the cytotoxicity of PAMAM-NH₂ on HepG2 cells, smooth muscle cells (SMCs) and HUVECs (Human umbilical vein endothelial cells) [14]. Espanha et al. in Brazil, using the Ame's test on *S. typhimurium* TA100, TA98, TA97a and TA102, with (+S9) and without (-S9) metabolization, reported the Mutagenic and antimutagenic activity of the plant *Byrsonima* that is widely used to treat gastrointestinal complications [15]. Dressler et al. in Germany determined the biological potency of the Botulinum toxin through an LD₅₀ assay [16]. In this study, we survey the mutagenic and toxicogenic properties of 4 generations of PAMAM-NH₂ (G2.0, G3.0, G4.0, G5.0) by making use of MTT assay and Ame's test and also, we determined the LD₅₀ of the generations of PAMAM-NH₂.

Materials and Methods

Toxicity study

The cytotoxicity of PAMAM-NH₂ (G2.0, G3.0, G4.0, and G5.0) (Source of this synthetic polymers was supplied from Amirkabir University of Technology (Fig. 1)) to human mesenchymal stem cells (MSCs), human gastric adenocarcinoma (AGS) cells and BALB/c mice was determined by a standard MTT assay and Mean lethal dose (LD₅₀) test.

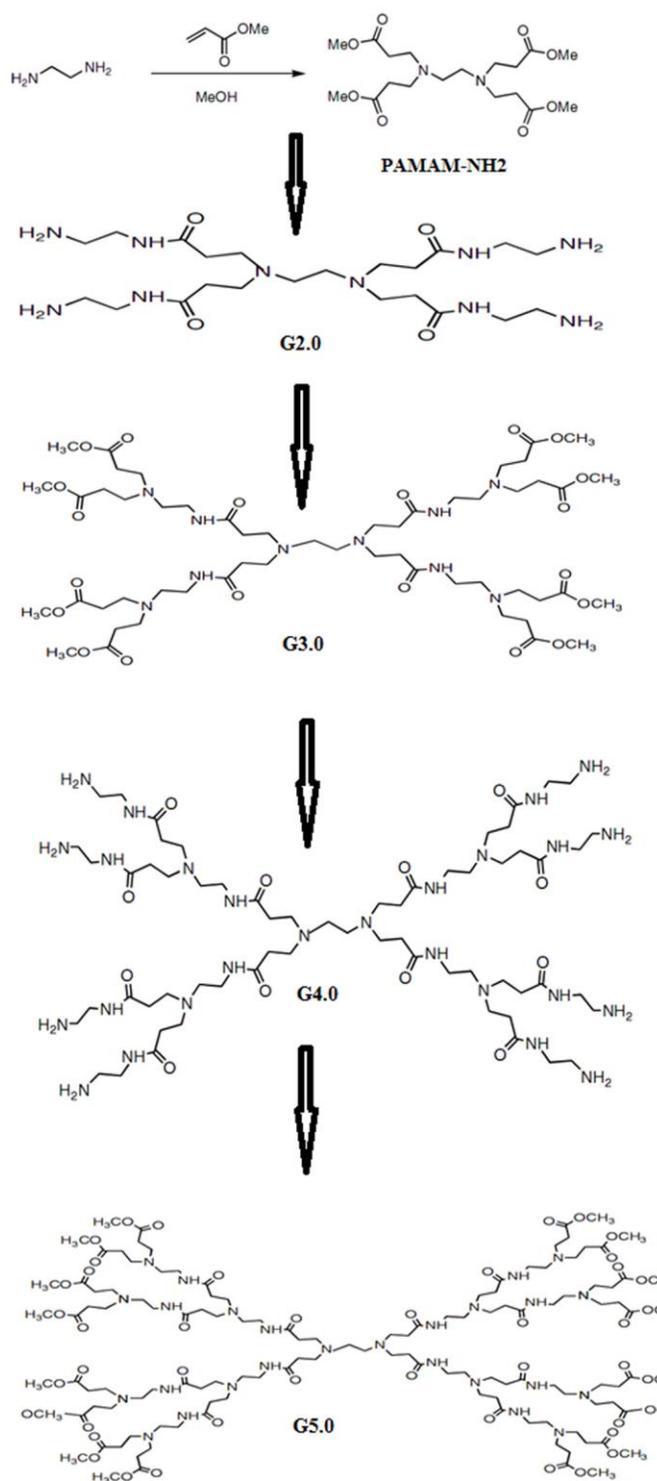


Fig.1. Structure and at least scheme of polymer

MTT assay

MSCs were cultured in Dulbecco's modified Eagle's medium media (DMEM) (Grand Island, NY) with 10% fetal bovine serum (FBS) (GIBCO, USA) and 30 µg/ml gentamicin (Sigma, USA). Also AGS cells were cultured in the Roswell Park Memorial Institute medium (RPMI) (Sigma, USA) with 10% FBS. Cells (10×10^3 /in each well) were grown for 24 h at 37°C in 96-well plates. The media containing the FBS was removed, and the cells were washed with 1x phosphate-buffered saline (PBS) and serum-starved for 4h and then, were cultured in DMEM and RPMI media and incubated with 5% CO₂ in the temperature of 37°C. The medium in the plate was replaced with the dilution of 0.01(W/W) (1µl of each of G2.0, G3.0, G4.0, and G5.0 that were mixed with 99 µl of the medium), 0.001 (W/W) (1µl of each of G2.0, G3.0, G4.0, and G5.0 that were mixed with 999 µl of the medium), and 0.0001(W/W) (1µl of each of G2.0, G3.0, G4.0, and G5.0 that were mixed with 9999µl of the medium) of a prepared PAMAM-NH2 solution. Then, the plate was incubated at 37°C for 48 h according to the MTT assay Kit's protocol. The absorbance was read at 490 nm (Bio Idea, Iran).

Mean lethal dose (LD₅₀)

125 Male and female BALB/c mice (8–10 weeks of age, weighing approximately 20 g) were used in this study. 5 mice as the control group and 5 mice as the examined group were used for each generation (G2.0, G3.0, G4.0, and G5.0) and each dose of PAMAM-NH2 (30, 47, 73.5, 115 and 180 mg/kg). The LD₅₀ was determined by the Spearman–Karber method. Animals were injected intraperitoneally with PAMAM-NH2 G2.0, G3.0, G4.0, and G5.0 that were dissolved in 0.01 M PBS with the pH of 7.4, at doses of 30, 47, 73.5, 115 and 180 mg/kg. Control animals also were injected by saline but didn't receive PAMAM-NH2. The animals were observed for a period of 4 h after injection with the purpose of observing any abnormal behavior such as changes in horizontal or vertical motion, level of activity, and eating or drinking behaviors. The survival rate of each

concentration group was observed for 7 days and recorded to calculate the LD50. The percentage of survival for each concentration that was injected to the groups was observed for 7 days and the percentage of survival of the mice was recorded to calculate the LD50 [17].

Mutagenicity study

In this study, the Ame's test was used as a biological test for detection of mutagenic potential of PAMAM-NH2 (G2.0, G3.0, G4.0, and G5.0).

Ame's test

The applied media for the Ame's test were Glucose minimum agar (10% glucose), Top agar (Sigma, USA) with 0.005 mM biotin-histidine and nutrient broth (Merck, Germany) [18]. The *S. typhimurium* TA100 strain was used for this study [18, 19]. For the Ame's test in the presence of rat microsomal liver enzyme (+S₉), fresh overnight cultures of *S. typhimurium* mutants (TA100), for cell cycle; 0.005 mM histidine -biotin solution and liver microsomes (+S₉) were added to 4 separate test tubes each containing 3 ml of Top agar (tube 1 contained diluted water as negative control, tube 2 contained 50µL of different generations of PAMAM-NH2 (G2.0, G3.0, G4.0, and G5.0) that were divided into 4 separate tubes, tube 3 contained sodium azide as positive control and tube 4 contained DMSO as negative control), then these materials after being shaken evenly were spread on Glucose minimal medium. In the next step, the medium was incubated at 37°C for 24 h (Table 1) [18]. Afterward, the Ame's test in the absence of rat microsomal liver enzyme (-S₉) was performed, similar to the procedure that was described above (Ame's test in the presence of rat microsomal liver enzyme (+S₉)), but in this step no liver microsomes were added [18].

Statistical analysis

All statistical analyses were performed using SPSS 16 (SPSS Inc., Chicago, IL). Data were subjected to analysis of variance (ANOVA) and Kruskal-Wallis test at the level of $p < 0.05$.

Results

The cell viability was determined using the MTT assay. The concentration of the PAMAM-NH2 is plotted in figures 1 and 2. Results of PAMAM-NH2 cytotoxicity in both cell lines showed that in every

generation, cytotoxicity was increased with increasing the dilution and generation. Among these four generations (G2.0, G3.0, G4.0 and G5.0), G2.0 and G3.0 of PAMAM-NH2 showed minimal cytotoxicity and G4.0 and G5.0 exhibited maximum cytotoxicity on both cell lines (Fig. 2, 3).

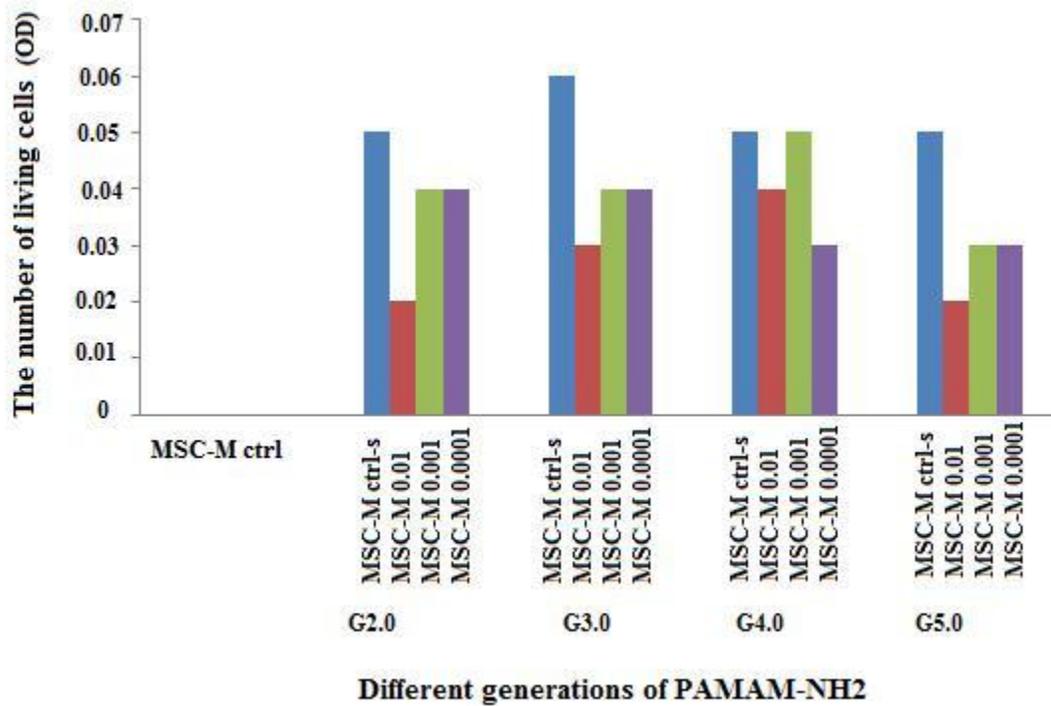


Fig.2. PAMAM-NH2 generations' toxicity in human MSCs cell lines at different dilutions (0.01, 0.001 and 0.0001 (W/W)). MSCs: Mesenchymal stem cells, MSC-M ctrl: MSC-Media (for cell culture) control, MSC-M ctrl-s: MSC-Media control-solution (Methanol), OD: Optical Density.

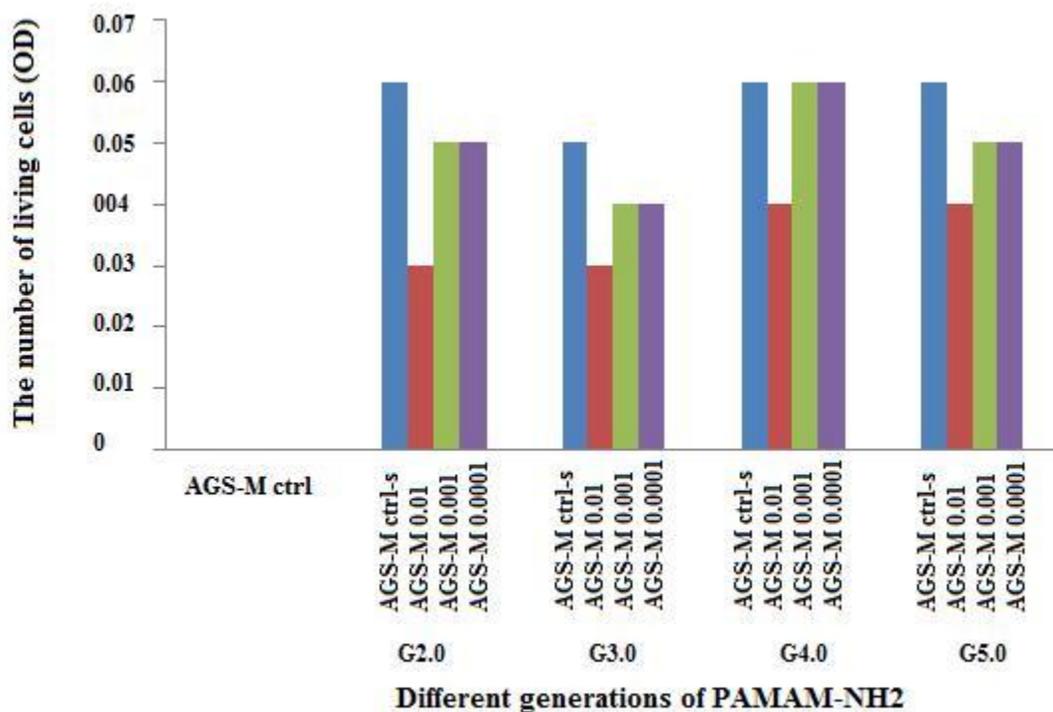


Fig. 3. PAMAM-NH2 generations' toxicity in AGSs cell lines at different dilutions (0.01, 0.001 and 0.0001 (W/W)). AGSs: Gastric adenocarcinoma cells, AGS-M ctrl: AGS- Media (for cell culture) control, AGS-M ctrl-s: AGS-Media control-solution (Methanol), OD: Optical Density.

Symptoms of toxicity were observed in doses of 73.5, 115 and 180 mg/kg in all the generations. These symptoms were abnormal behavior in eating and drinking, twitching, stiffness, urinary and fecal incontinence that were observed in less

than 3h. All the mice died within 24 h. LD₅₀ of PAMAM-NH2 generations were in the 73.5 mg/kg (Fig. 4).

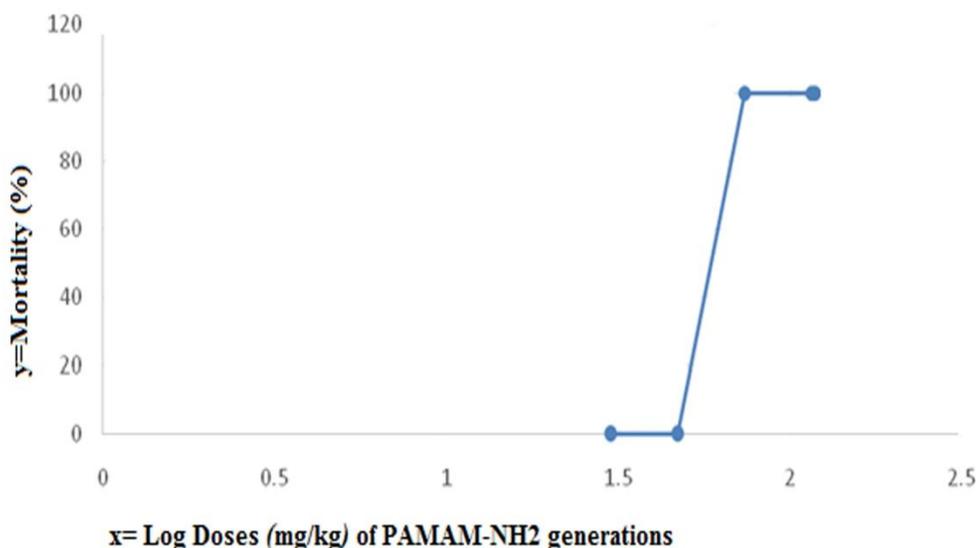


Fig. 4. LD50 curve of PAMAM-NH2 generations

Results of the Ame's test Showed that the numbers of reverted colonies were increased by increasing the generation and significant differences were observed in doing so. Inhibition percentages of PAMAM-NH₂, that were used in the Ame's test were 69.47%, 68.42%, 64.210% and 64.21% for the PAMAM-NH₂ G2.0, G3.0, G4.0 and G5.0, respectively. Statistical analysis of ANOVA showed significant differences for all of the returned colonies. The maximum difference between the averages of returned colonies was related to sterile distilled water and sodium azide with the number of 1522.64 colonies and p- Value of 0.001. Between different generations of PAMAM-NH₂, the least difference was related respectively to PAMAM-NH₂ G2.0, G3.0, G4.0 and G5.0 in comparison with sterile distilled water and DMSO in terms of the number of returned colonies. P- Value for PAMAM-NH₂ G2.0 was 0.01 and the mean of returned colonies was 65.95. Also the different generations of PAMAM-NH₂ showed no significant difference regarding the number of returned colonies. But the Kruskal-Wallis test showed a significant difference between sodium azide and other tested materials. Also a significant difference was observed between the test and control groups ($p < 0.05$).

Discussion

Amino-terminated PAMAM-NH₂ generations showed the strongest antibacterial activity in *in-vitro* studies. It is used in cancer treatment and delivery of different drug molecules to the cells [20, 21]. For this reason, many researchers in recent years have applied dendrimers and dendritic structures in medicine, pharmacy, drug delivery systems, stem cells and tumor therapy in cancer cells [21, 22]. However, studies have shown that PAMAM-NH₂ has cytotoxicity and genotoxicity effects on cells [20, 23]. Results of our study showed the PAMAM-NH₂ generation's cytotoxicity in both cell lines and toxicity symptoms including abnormality in behavior and eventual death in mice. LD₅₀ determination in our results indicated the toxicity of PAMAM-NH₂ in doses of 73.5, 115 and 180 mg/kg in all the generations. Also reverted colonies were increased by increasing the generation in the Ame's test. Li et al. in 2005 in China showed the cytotoxicity effect of pseudolaric acid B on AGS by the MTT test. Results of MTT assay in our study showed the cytotoxicity of PAMAM-NH₂ in MSCs and AGS. These results indicated that different chemicals such as

PAMAM-NH₂ and pseudolaric acid B disrupt cells and they can dramatically suppress the cell growth by inducing apoptosis and activating protease such as caspase-3^[24]. PAMAM-NH₂ also may damage the cells through nonspecific physical mechanisms rather than by targeting specific molecules^[20]. Hamidi et al. in 2012 in Iran using the MTT test, showed the cytotoxicity of G1 and G2 of PAMAM-NH₂ on the MCF-7 (Michigan Cancer Foundation-7) or human breast adenocarcinoma cell line. Based on our study, G2.0 and G3.0 had minimal cytotoxicity and G4.0 and G5.0 had maximum toxicity. Higher-generation PAMAM-NH₂ dendrimers had stronger cytotoxicity, also studies showed that cytotoxicity of PAMAM dendrimers depends on the generation, size, their surfaces, concentration, type of cells and condition of reaction^[25]. Partoazar et al. in 2009 using the Ame's test on 57 urine samples gathered from the personnel's of a medical laboratory in Iran, showed that the levels of mutagenicity by *S. typhimurium* (TA98) without activator was in one case and with activator, in two cases of urine samples of pathology laboratory personnel^[26]. The *Salmonella* mutagenicity test is designed to detect chemicals that induce mutagenesis^[10]. In our study the Ame's test showed that different generations of PAMAM-NH₂ caused cytotoxicity in the cell lines and, reverted colonies were increased by higher generations of PAMAM-NH₂. The Ame's test has specific features such as sensitivity and specificity, so different materials can be used in this test^[27]. Pounikar and Dawande in 2010 by using the Ame's test showed that ethidium bromide, sodium azide, hair dye, colors and food additives had a mutagenic response on the auxotrophic strain (his-) of *S. typhimurium* and induced reverse mutation and as a result, turned *S. typhimurium* (his-) into prototrophic strain (his+) of *S. typhimurium*^[28]. Results of the Ame's test in this study showed that inhibition percentages of G2.0, G3.0, G4.0, and G5.0 were 69.47, 68.42, 64.210 and 64.21, respectively. Different materials that are used in the Ame's test may also produce a reproducible dose and increase the number of reverted colonies in one or more strains of *S. typhimurium*. Akyl and Konuk in 2014 in Turkey

showed pesticide mutagenicity by the Ame's test, but no concentrations of chlorthiophos showed any mutagenic activity on TA97, TA100, and TA102 strains of *S. typhimurium*. *S. typhimurium* strain TA100 was used for our study, because *Salmonella* strains have different mutations in various genes of histidine operon^[29]. Application of PAMAM in nanomedicine has increased, but according to results in our and other study, further investigations are needed to determine cytotoxicity and mutagenicity of PAMAM using other *in vivo* and *in vitro* biological test systems and usage of these materials must be with caution.

Conclusion

Results of our study showed that various generations of PAMAM-NH₂ had a cytotoxicity effect on MSC and AGS cell lines, also toxicity and mutagenicity of them were observed in mice and *S. typhimurium* bacterium respectively, and so they should not be used as drug carriers. Therefore, in order to use PAMAM-NH₂ in pharmaceuticals, we must be careful and appropriate methods must be used to ensure the safety of PAMAM-NH₂ generations.

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Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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